APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1986, p. 1014–1019
0099-2240/86/111014-06$02.00/0

Vol. 52, No. 5

Autecology in Rhizospheres and Nodulating Behavior of Indigenous *Rhizobium trifolii*†

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Received 8 April 1986/Accepted 4 August 1986

Indigenous serotype 1-01 of *Rhizobium trifolii* occupied significantly fewer nodules (6%) on plants of soil-grown noninoculated subterranean clover (*Trifolium subterraneum* L.) cv. Woogenellup than on cv. Mt. Barker (36%) sampled at the flowering stage of growth. Occupancy by indigenous serotype 2-01, was not significantly different on the two cultivars (16 and 26%). Serotype-specific, fluorescent-antibody conjugates were synthesized and used to enumerate the indigenous serotypes in host (clovers) and nonhost (annual rye-grass, *Lolium multiflorum* L.) rhizospheres and in nonplanted soil. The form and concentration of Ca^{2+} in the flocculating mixture and the presence of phosphate anions in the extracting solution were both critical for enumerating *R. trifolii* in Whobrey soil. The two serotypes were present in similar numbers in nonplanted soil (ca. 10^6 per g of soil) and each represented ca. 10% of the total *R. trifolii* population. Although host rhizospheres did not preferentially stimulate either serotype, the mean population densities of serotype 2-01 were significantly greater ($P = 0.05$) than those of serotype 1-01 in clover rhizospheres on 8 of 14 samplings made between the time of seeding and the appearance of nodules (day 12). In this experiment, and in contrast to our earlier findings, serotype 1-01 occupied significantly fewer ($P \leq 0.05$) of the nodules (7 to 16%) on both cultivars than serotype 2-01 (51%) when sampled at 4 weeks. Differences between cultivars became apparent as the plants matured. There was a threefold increase (7 to 21%) in nodules occupied by serotype 1-01 on cv. Mt. Barker between 4 and 16 weeks. This was accompanied by increases in nodules coinhabited by both nonidentifiable occupants and either serotype 1-01 (0 to 20%) or 2-01 (11 to 51%). No increases in either of these parameters were observed on cv. Woogenellup.

Many studies have been carried out over the past 30 years to elucidate those factors which influence the outcome of nodulation by mixtures of strains of *Rhizobium* species on legume hosts. A significant amount of this information has been accumulated from studying the *Rhizobium trifolii-Trifolium* (clover) species association (8, 23, 35–37). The results of these studies can be summarized as follows. Under laboratory conditions, using simple mixtures of *Rhizobium* strains, the relative abundance of the competing strains in the rhizosphere or rhizoplane zone or both was the major factor contributing to the outcome of nodulation (18, 25). Evidence was also obtained favoring the conclusion that the species of *Trifolium*, and even the cultivar within a species, can be the major factor affecting the outcome of nodulation. In this case the relative abundance of strains in the rhizosphere (17, 39) or in the inoculant mixtures (11, 15, 24, 30, 32) or in both was of minor consequence.

Neither of these explanations has been confirmed under soil conditions, nor has the influence of indigenous *R. trifolii* been evaluated. The significance of these deficiencies was recently demonstrated by reports of studies on the *Bradyrhizobium japonicum-Glycine max* association, where quantitative immunofluorescence was used to enumerate indigenous organisms in rhizosphere soil (21, 29). No evidence was obtained that either the relative abundance of different *B. japonicum* serogroups in the rhizosphere or the host plant variety influenced the overwhelming success of nodulation by members of serogroup 123 of *B. japonicum*. Previously, we reported on two indigenous serotypes of *R. trifolii* which were found in substantial portions of the nodules formed on field-grown noninoculated subterranean clover (*Trifolium subterraneum* L.) cv. Mt. Barker (9). Preliminary experiments indicated that the nodulating success of these two serotypes was influenced by the cultivar sown in this soil. The objectives of this study were (i) to develop an efficient method to extract and enumerate by immunofluorescence the population sizes of each of the two serotypes existing within a total *R. trifolii* population ranging between 10^5 and 10^6 per g of soil; (ii) to determine if host rhizospheres selectively stimulated the populations of the two serotypes between the time of seeding and the appearance of primary nodules; and (iii) to determine if the nodulating success of either serotype correlated with their relative abundance in the rhizospheres of two subclover cultivars, Mt. Barker and Woogenellup.

MATERIALS AND METHODS

Fluorescent-antibody preparation. Antisera were prepared against somatic antigens of *R. trifolii* strains 1-01 and 2-01, as previously described (9).

(i) Adsorption of antisera. Prior to the synthesis of the fluorescent antibodies, each antiserum was adsorbed with whole cells of the heterologous antigen. This was necessary due to a small, yet significant amount of cross-reactivity between each antiserum and the heterologous antigen (9). Adsorption of antiserum 2-01 was accomplished as described elsewhere (13). A total of nine adsorption cycles, using a fresh batch of cells for each cycle, was necessary to eliminate completely the cross-agglutinating reactivity of antiserum 2-01 with heat-treated whole cells of strain 1-01. After

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adsorption was completed, the antisera were sterilized by passage through polycarbonate membranes (0.4-μm pore size; Nuclepore Corp., Pleasanton, Calif.) prior to continuing with the conjugation procedure.

(ii) Chromatographic and calibration procedures. Fluorescein-labeled immunoglobulin conjugates were prepared as described elsewhere (2). The fluorescein-labeled immunoglobulin conjugates were characterized to determine working dilution (ranging from 1/10 to 1/50 dilution of conjugates) and serotype specificity on smears from both broth-grown cultures of, and nodules formed by, a variety of antigenically distinct *R. trifolii* isolates.

Development and evaluation of methods for enumerating indigenous *R. trifolii* in Whobrey soil. A protocol was developed to optimize the efficiency of extraction and enumeration of indigenous *R. trifolii* from the Whobrey soil. The physical and chemical characteristics of the latter have been described in detail elsewhere (9). Portions of moist soil (8 g, oven dry weight) were dispersed by the procedure of Kingsley and Bohlool (16) with the minor modifications of placing 50 ml of extracting solution (ES) and 5 g of glass beads (3-mm diameter) with the soil in each 160-ml milk dilution bottle. The bottles were then shaken for 15 min on a wrist-action shaker (Burrell Corp., Pittsburgh, Pa.), after which another 45 ml of ES was added and the suspensions were shaken for an additional 15 min.

(i) Optimizing the composition of the flocculating mixture. Preliminary experiments showed that the $Ca(OH)_2$-$MgCO_3$ mixture, commonly used by others (16), was ineffective in flocculating Whobrey soil. Any one of several Ca^{2+} salts more soluble than $Ca(OH)_2$ was superior. However, the form and concentration of these were critical. Propionate and acetate salts resulted in the bulk density of the flocculated material being so low that insufficient supernatant could be recovered (data not shown). Although calcium chloride was superior in this regard, the amount of Ca^{2+} was critical if effective flocculation and optical background of the membrane filters were to be satisfactory. Varying amounts of solid $CaCl_2 \cdot 2H_2O$, ranging from 0.26 to 1.3 g, were thoroughly mixed with 0.5-g portions of $MgCO_3$ [$4MgCO_3 \cdot Mg(OH)_2 \cdot 4H_2O$]. Each mixture was tested for its ability to flocculate the soil colloids by adding it to 10-g portions of soil already dispersed as described above, followed by shaking for another 2 min on the wrist-action shaker. After the samples had been allowed to flocculate for 1 h, portions of the cleared supernatants were passed through Irgalan black-treated polycarbonate filters (12), supported on silver metal membranes (0.8-μm pore size; Selas Corp., Huntingdon Valley, Pa.) as originally described by Zimmerman et al. (41). The membranes were examined under ×900 magnification to determine the optical quality of the background for immunofluorescence analysis. A mixture of 5.5 mmol (0.8 g) of $CaCl_2 \cdot 2H_2O$ and 0.5 g of $MgCO_3$ was found to be optimum and was used in all analyses described below.

(ii) Evaluating the composition of the ES. The composition of the ES influenced the flocculating behavior and subsequent enumeration of indigenous *R. trifolii* (Table 1). Different ES, amended with partially hydrolyzed gelatin to a final concentration of 0.1% (wt/vol), were examined quantitatively for both their effects on flocculation of the Whobrey soil and the efficiency of extraction of indigenous *R. trifolii*. The solutions included 0.1 M $(NH_4)_2HPO_4$, 0.1 M K_2HPO_4, 0.1 M Na_2HPO_4, 0.2 M NH_4Cl, 0.2 M NH_4 acetate, 0.1 M $(NH_4)_2$ oxalate $\cdot H_2O$, and distilled water. All of the solutions were adjusted to pH 8.2 with either 1 M NaOH or 1 M HCl. Duplicate 10-g samples of moist soil were placed into milk dilution bottles along with the various ES and dispersed as described above. One hour after initiating flocculation with $CaCl_2$-$MgCO_3$, the turbidity of the supernatants was measured at 660 nm, portions of those supernatants which had been completely, or partially, cleared were filtered, and *R. trifolii* were enumerated as described below. A critical role for the phosphate anion in flocculation is apparent since the optical densities of the flocculated supernatants of the three phosphate salts were very low, and similar values for the enumeration of serotype 2-01 were obtained (Table 1). Soil samples dispersed in either non-phosphate-containing ammonium salts or distilled water were poorly flocculated. These suspensions were either impossible to filter or, in the case of NH_4Cl, left a deposit on the membrane which resulted in substantially lower cell counts. Although the data showed that any monohydrogen phosphate salt would be adequate, ammonium monohydrogen phosphate was used in all studies described so that our procedure would not be modified unnecessarily from that already published (16).

(iii) Determining influence of soil quantity and plant rhizoplane on the efficiency of recovery and enumeration of *R. trifolii*. An experiment was carried out in which various amounts of soil, 1 to 10 g, were extracted as described above. A linear relationship ($y = 3.8 + 8.4x$; $r^2 = 0.98$) was established between the numbers of cells counted per field of view (y) and the weight of soil extracted (x). The extraction procedure was found to be very effective at enumerating rhizosphere/rhizoplane populations since only 0.4% of the total *R. trifolii* determined in this zone were still associated with the roots of seedlings after extraction. It was also determined that both indigenous serotypes 1-01 and 2-01 could be recovered at 10% efficiency from the Whobrey soil. These experiments are described in detail elsewhere (D. H. Demezas, Ph.D. thesis, Oregon State University, Corvallis, 1986).

(iv) Immunofluorescence analysis. Membranes were treated with gelatin-rhodamine conjugate to suppress nonspecific fluorescence (5), stained for 1 h with appropriately diluted prefiltered fluorescein conjugate, and destained by drawing 100 ml of 0.02 M phosphate buffer, pH 7.3, under 50 kPa of negative pressure through each of them. The membranes were placed on clean microscope slides, covered with buffered glycerol (pH 7.2; Difco Laboratories, Detroit, Mich.), and viewed with a Zeiss standard microscope (Carl Zeiss Inc., New York, N.Y.) equipped with a 100-W tungsten halogen lamp and operated in the epi-illumination mode. The filter set was a Zeiss fluorescein isothiocyanate filter pack consisting of a fluorescein excitation filter BP 450-490, an FT 510 beam splitter, and an LP 520 barrier filter. A Zeiss Planachromat 100×/1.25 oil objective was used for all observations. For enumeration purposes, a minimum of 20 fields of view were counted per filter; 40 fields were counted where less than an average of five cells per field was encountered. The mean of each sample was determined, and the values for the number of bacteria per gram of oven-dry soil were calculated.

Influences of subclover cultivars on nodule occupancy by indigenous *R. trifolii*. Five replicate pots (7.2 by 21 cm), each containing the equivalent of 0.77 kg of air-dried Whobrey soil, were sown with surface-sterilized seed of both subclover cv. Mt. Barker and Woogenellup. After germination, each pot was thinned to three healthy plants of each cultivar. The soil was moistened to, and maintained at, 20- to 33-kPa water potential throughout the experiment. The plants were maintained under growth conditions previously

described (9) and were harvested after 56 days of growth (early flowering stage). All nodules were collected from each plant within each replicate and surface sterilized by standard methods (38). Ten nodules from each plant were homogenized separately in distilled water and 5-μl portions were spotted onto duplicate clean slides. The smears were air dried, gently heat fixed, and examined by immunofluorescence as described above.

Evaluation of the population dynamics of indigenous *R. trifolii* in host and nonhost rhizospheres between the time of seeding and appearance of nodules. Four replicate boxes (0.6 by 1.8 m) were filled with a layer of coarse river sand (7.6 cm) and overlaid with Whobrey soil (30 cm). The soil was moistened to, and maintained between, 20- and 33-kPa water potential for 10 days prior to seeding under the greenhouse conditions described above. Surface-sterilized seeds of subclover cv. Mt. Barker and Woogenellup were imbibed in sterile distilled water for 24 h at room temperature. Annual ryegrass (*Lolium multiflorum* L. cv. Marshall; courtesy of D. Chilcote, Oregon State University) and the two subclover cultivars were sown in three separate bands 15 cm wide along the full length of the box with 5 cm between the bands. Each box was considered a replicate and the bands of seed were randomized within each replicate. The seeds were covered with a fine layer of sieved soil (6 mm), and the soil was watered to field capacity (33 kPa) when necessary. Nonplanted soil was maintained under the same greenhouse conditions and at the same water potential to serve as a control.

Approximately 100 seedlings and 10-g samples of nonplanted soil were collected from each treatment replicate at 2-day intervals from the time of sowing to day 18. Thereafter seedlings and soil samples were taken at 34, 56, and 112 days after sowing. The intact seedlings were shaken gently and the soil still adhering was considered rhizosphere soil (26). The samples of seedling roots and nonplanted soil were transferred into 160-ml screw-cap milk dilution bottles and treated as described above to extract and enumerate serotypes 1-01 and 2-01 of *R. trifolii*. In some rhizosphere samples (ryegrass in particular), a crystalline precipitate accumulated upon the membranes after filtration. To dissolve this material, the membranes were treated with 1% (vol/vol) lactic acid (Sigma Chemical Co., St. Louis, Mo.) for 10 min and then rinsed with 10 ml of 0.02 M phosphate buffer (pH 7.3). This treatment had no effect on the staining properties of cells of either serotype which were enumerated as described above. The total *R. trifolii* populations relative to the total bacterial populations in host and nonhost rhizospheres were determined by extracting soil from the rhizospheres of 20-day-old plants and serially diluting fourfold in 0.15 M phosphate-buffered saline over a range of 4^1 to 4^7. Portions (1 ml) of each dilution were inoculated onto four subclover cv. Mt. Barker seedlings. After 28 days of growth in the greenhouse, nodulation was assessed and the most probable numbers of viable *R. trifolii* per gram of rhizosphere soil were determined (38). In addition, 0.1-ml portions of each dilution were plated onto duplicate plates of 1/10-strength tryptic soy broth, solidified with 1.5% (wt/vol) agar (Difco Laboratories) and amended with 0.002% (wt/vol) cycloheximide (Sigma Chemical Co.). After 48 h of growth the total viable bacterial count per gram of oven-dry soil was determined. At 4, 8, and 16 weeks after sowing, between 20 and 25 nodules were removed from the roots of plants from each replicate at each of the three sampling dates. Nodule occupancy was determined by immunofluorescence as described above.

TABLE 1. Influence of ES composition on flocculation of Whobrey soil and enumeration of indigenous *R. trifolii*[a]

ES[b]	OD_{660}[c]	No. of serotype 2-01 per g of dry soil[d]
0.1 M Na_2HPO_4	0.06	0.97×10^6
01.1 M K_2HPO_4	0.04	0.83×10^6
0.1 M $(NH_4)_2HPO_4$	0.03	0.91×10^6
Distilled water	0.12	ND
0.2 M NH_4Cl	0.132	0.58×10^6
0.2 M NH_4 acetate	0.26	ND
0.1 M $(NH_4)_2$ oxalate	>1.5	ND

[a] See Materials and Methods for complete details.
[b] Adjusted to pH 8.2; all contained partially hydrolyzed gelatin (final concentration, 0.1%, wt/vol).
[c] Optical density of the supernatant at 660 nm (OD_{660}) above flocculated soil measured through a 1-cm-path length cell 1 h after initiating flocculation.
[d] Mean of duplicate samples; fluorescent-antibody conjugate to serotype 2-01 was used in this experiment. ND, Not determined.

RESULTS

Significantly more nodules were found on plants of cv. Woogenellup than on those of cv. Mt. Barker (Table 2). Nodules from cv. Mt. Barker and Woogenellup were analyzed by using the fluorescent-antibody conjugates available. On cv. Mt. Barker, serotypes 1-01 and 2-01 were present in similar percentages of nodules, confirming previous observations made on field-grown plants of this cultivar (9). In contrast, analysis of nodules from cv. Woogenellup showed that, although the percent occupancy by serotype 2-01 was not significantly different on the two cultivars, the occupancy by serotypes 1-01 was significantly less than on cv. Mt. Barker. Indeed, although cv. Woogenellup plants had significantly more nodules, 50% of these plants were completely devoid of serotype 1-01, whereas only 7% of cv. Mt. Barker plants were not occupied by serotype 1-01. The number of unidentifiable nodule occupants was significantly greater on cv. Woogenellup than on cv. Mt. Barker.

Prior to carrying out the rhizosphere study, the experiments described in detail in the Materials and Methods section were the essential prerequisites for enumerating *R. trifolii* in Whobrey soil. In addition, one of the major criticisms of enumeration by immunofluorescence is that the technique cannot discriminate between viable and nonviable cells. The numbers of serotypes 1-01 and 2-01 determined by immunofluorescence in several soil samples were similar (0.4

TABLE 2. Nodulation of subclover cv. Mt. Barker and Woogenellup by two serotypes of *R. trifolii* indigenous to Whobrey soil

Cultivar	No. of nodules per plant	No. of nodules analyzed	%[a] of serotype: 1-01	2-01	Others
Mt. Barker	22	134	36 (19)	26 (9)	47
Woogenellup	42	160	6 (4)	16 (1)	79
	**[b]		**[c]	NS[d]	**[c]

[a] Mean percentage of nodules occupied by the particular serotype in five replicated pots. Plants sampled at flowering stage (56 days). Percentages reported include singly occupied and coinhabited nodules; the parenthetical value reports the percentage of the total nodules analyzed which were coinhabited.
[b] One-way analysis of variance was carried out on the average number of nodules per plant from the five replicated pots (**$P \leq 0.01$).
[c] One-way analysis of variance was carried out on the arcsine $\sqrt{\%}$ transformed data of five replicates (**$P \leq 0.01$).
[d] NS, Not significant.

$\times 10^6$ to 0.6×10^6 per g of oven-dry soil) and correlated with values established by detecting the two serotypes in nodules of the final positive dilution of a soil dilution-plant infection technique (data not shown).

The population dynamics of the two serotypes in host and nonhost rhizospheres, and in nonplanted soil, from the time of seeding to the appearance of nodules are presented in Fig. 1. With the exception of four erratic values for serotype 2-01 (Fig. 1C and D), the variation within the four replicates of each treatment sample was modest. The population sizes of the two serotypes were large in the nonplanted soil and, when correlated for efficiency of recovery, ranged between 5×10^5 and 1×10^7 per g of oven-dry soil. No evidence was obtained for rhizosphere stimulation or for a differential one. With the exception of three samplings, the mean population of serotype 2-01 was greater than 1-01 and significantly greater ($P = 0.05$) than serotype 1-01 in clover rhizospheres on 8 of 14 samplings made between sowing the seed and the appearance of nodules at day 12 (Fig. 1A and B). A similar trend was apparent in nonplanted soil for four of seven samplings (Fig. 1D); on only one sampling date was such a trend observed for ryegrass rhizospheres (day 12). Furthermore, in the latter rhizosphere it was repeatedly observed that ≥90% of the rhizobia cells were ≤1.9 μm in length, whereas in clover rhizospheres ≥90% of the cells were ≥2.8 μm in length (data not shown).

Twenty-four days into the experiment, the viable populations of both soil bacteria in general and total *R. trifolii* were determined for each of the three rhizosphere treatments (Table 3). No evidence was found for differential stimulation of these populations in the three rhizospheres. The *R. trifolii* values are similar in magnitude to those of the two serotypes measured by immunofluorescence (Fig. 1) and account for between 0.09 and 0.68% of the total bacterial population of the rhizospheres.

TABLE 3. Rhizosphere populations of *R. trifolii* and total enumerable bacteria after 20 days in the presence of the roots of host or nonhost plants

Rhizosphere	No. of organisms per g of dry soil[a]		*R. trifolii*/total bacteria (%)
	Total bacteria[b]	Total *R. trifolii*[c]	
Ryegrass	2.8×10^8	2.2×10^5	0.09
Mt. Barker	5.4×10^8	8.8×10^5	0.16
Woogenellup	1.3×10^8	8.8×10^5	0.68

[a] Values are not corrected for efficiency of soil extraction.
[b] Viable count determined on 1/10-strength tryptic soy broth solidified with 1.5% (wt/vol) agar.
[c] Most probable number of *R. trifolii* determined with cv. Mt. Barker as the host plant (38).

At the 4-week sampling date, and in contrast to earlier findings (Table 2), serotype 1-01 occupied significantly fewer ($P \leq 0.05$) nodules than serotype 2-01 on both cv. Mt. Barker and Woogenellup (Table 4). By week 8 of growth, the percentages of nodules occupied by serotypes 1-01 and 2-01 had increased on cv. Mt. Barker and the majority were coinhabited by other unidentifiable organisms. These phenomena did not occur on cv. Woogenellup, where the occupancy by serotype 1-01 and the incidence of coinhabited nodules remained low.

DISCUSSION

Our preliminary observations (Table 2) provided a rationale to study the relative abundance of the two indigenous serotypes in soil and to determine whether or not the

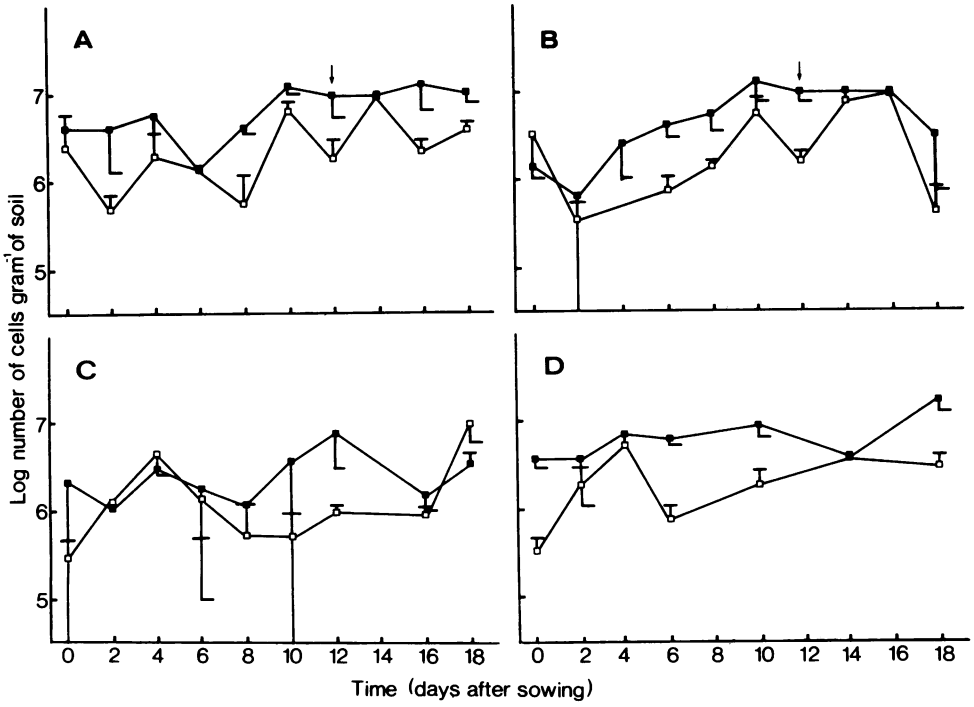

FIG. 1. Population densities of *R. trifolii* serotypes 1-01 and 2-01 in both subclover and ryegrass rhizosphere and nonplanted soil: (A) subclover cv. Mt. Barker; (B) subclover cv. Woogenellup; (C) ryegrass cv. Marshall; (D) nonplanted soil. Symbols: □, serotype 1-01; ■, serotype 2-01. Bars indicate 95% confidence limits based on four observations; ⊤ represents the limit for the lower of the two means and ⊥ represents that for the higher of the two means on a particular sampling date. Arrows indicate time when nodules were first observed.

TABLE 4. Nodule occupancy by serotypes 1-01 and 2-01 on cv. Mt. Barker and Woogenellup over the life cycle of the host plants

Cultivar	Sample time (wk)	No. of nodules analyzed	%[a] of serotype:		
			1-01	2-01	Others
Mt. Barker	4	85	7 (0)	51 (11)*[b]	46
Woogenellup		94	16 (4)	51 (23)*	36
Mt. Barker	8	95	17 (15)	79 (54)*	19
Woogenellup		93	9 (7)	62 (13)*	38
Mt. Barker	16	97	21 (20)	69 (51)NS	28
Woogenellup		76	7 (2)	38 (11)*	59

[a] Mean percentage of nodules occupied by serotypes 1-01 and 2-01 from four replicates. Parenthetical values report the percentage of the total nodules analyzed in which the particular serotype was a co-occupant.

[b] One-way analysis of variance was carried out on the arcsine $\sqrt{\%}$ transformed data from each sampling date for each cultivar (*$P \leq 0.05$). NS, Not significant.

different outcome of nodulation on the two cultivars was related to differential stimulation of the two serotypes in the cultivar rhizospheres. Sporadic reports in the literature on quantitative immunofluorescence of rhizobia in soils suggest that the first two steps, extraction and flocculation, can affect the efficiency of recovery of added or indigenous cells (4, 16, 28, 33, 40). Kingsley and Bohlool (16) formulated and successfully used 0.1 M $(NH_4)_2HPO_4$ amended with 0.1% (wt/vol) partially hydrolyzed gelatin to extract and enumerate rhizobia added to both tropical and temperate soils. The success of this extracting solution was attributed to the commonly accepted roles of the phosphate and ammonium ions in saturating the anion- and cation-exchange sites, respectively, of clay minerals (3, 22, 27), which might be implicated in the binding of rhizobia. In Whobrey soil no specific role could be ascribed to the phosphate anion or associated cation in the extraction of the rhizobia from this soil (Table 1). Our data suggest that the critical role of the phosphate anion is simply to provide a coprecipitable counteranion for calcium in the flocculation process. We have recently determined that a combination of ammonium hydroxide as extractant and $CaCl_2$ as flocculant, originally described elsewhere (28), is also an effective flocculating combination for this soil (data not shown). However, on a cautionary note, illustrating the complexity of soils, addition of the flocculating mixture to soil suspended in ammonium oxalate had no impact on the dispersed state of the soil (Table 1). Studies in our laboratory with other acidic (≤pH 5.3) pasture soils, generally fine textured, containing much organic matter (≥6%, wt/wt), and of the Ultisol, Mollisol, and Inceptisol orders have shown this procedure to be generally satisfactory. The quantity of $CaCl_2$ in the flocculating mixture does require fine adjustment for each soil type.

Members of both serotypes were found to be capable of persisting within Whobrey soil in high numbers, with each type representing at least 10% of the total *R. trifolii* population. The size of this indigenous population was high, considering the soil was sampled in summer (August) when the clover was dead and the pasture had completely dried out. These data add support to the concept of Schmidt and Robert (34) that *Rhizobium* spp. should be accepted as bona fide soil saprophytes. Other workers have also shown the population sizes of different indigenous serogroups of *B. japonicum* in the same soil to be similar (21, 29). In addition, the percentages (0.09 to 0.68%) that *R. trifolii* contributed to the total rhizosphere bacterial population (Table 3) were in close agreement with values reported for *B. japonicum* (21). They are much lower than a value for total *R. trifolii* of 14% reported for rhizospheres of 12-week-old red clover plants growing in an acid soil (31). The latter value could have been influenced by release of rhizobia from senescing nodules, a phenomenon recently observed on field-grown plants of soybean (21) and *Vigna* sp. (7). In our studies, no evidence of nodule senescence or of extensive rhizosphere proliferation was observed even after 16 weeks when the plants were setting seed. At that time, populations of 1.8×10^6 and 1.9×10^6/g for 1-01 in cv. Mt. Barker and Woogenellup rhizosphere soil, respectively, and 9.6×10^6/g for 2-01 in both cultivar rhizospheres were observed. The plants used in our study were well watered and certainly not suffering the drought stress they would normally encounter in early summer field conditions at the time of setting seed.

Although there was no selective rhizosphere proliferation, we repeatedly found that serotype 2-01 did outnumber serotype 1-01 in the Whobrey soil (Fig. 1). It is possible that the excess of 2-01 did account for the superior nodulation of 2-01 over 1-01. The findings may, however, have been serendipitous since others have observed similar differences between the rhizophere populations of indigenous serogroups 123 and 110 of *B. japonicum* and considered them to be insignificant to the outcome of nodulation (21).

There was an obvious discrepancy between the low occurrence of serotype 1-01 in primary nodules on cv. Mt. Barker observed in the rhizosphere experiment (Table 4) and its more extensive occupancy of nodules at flowering in the preliminary experiment (Table 2) and our earlier field data (9). Both the increase in the incidence of coinhabited nodules and the increase in percent occupancy by serotype 1-01 with time on plants of cv. Mt. Barker might provide clues. In the former case, the literature on the subject of multioccupied nodules reports values typically between 15 and 25% for various *Rhizobium*/legume associations (6, 14, 19). Our values are similar to somewhat higher percentages reported recently for highly competitive strains of *R. leguminosarum* in nodules of soil-grown lentils (20). It is apparent that extensive occurrence of coinhabited clover nodules need not necessarily be an artifact of nonsoil-rooting media but can be significant on soil-grown plants. Regarding nodule occupancy by serotype 1-01 changing with time, Bushby (7) has made similar observations on the behavior of inoculant strains on *Vigna* sp. He advises against single nodule harvests if nodulating success of rhizobia is being evaluated. Furthermore, since members of serotype 1-01 are more effective than those of 2-01 (9), and since cultivars of legumes can vary with respect to the stage of their life cycle where the majority of N_2 is fixed (1), we have given these findings more attention (10).

ACKNOWLEDGMENTS

This research was supported by funds from U.S. Department of Agriculture–Science and Education competitive grants 81-CRCR-1-0726 and 85-CRCR-1-1704 and the Oregon Agricultural Experiment Station.

We thank P. Tenbrook and A. Almendras for invaluable assistance and C. Pelroy for typing the manuscript.

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